Cloning and expression analysis of the hexamerin subunit type 2 (Hex2) gene from the grasshopper *Calliptamus italicus* (Orthoptera: Catantopidae)

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Abstract: 【Aim】 Hexamerins are large hemolymph-proteins that accumulate in insects during the late larval stages and serve important roles throughout insect development and growth. 【Methods】 Our study cloned the hexamerin subunit type 2 (Hex2) gene from the grasshopper *Calliptamus italicus* and by using qRT-PCR method analysed its expression patterns in different tissues and at different developmental stages. 【Results】 The cDNA of *C. italicus Hex*2 (*CitHex*2) is 2 610 bp in length, containing a 2 022 bp open reading frame (ORF) and a 124 bp 3′ untranslated region (UTR). BLAST analysis showed that it shares 80% – 84% nucleotide sequence identity with Hex2 genes from other grasshoppers. The encoded protein was predicted to be 673 aa in length with an estimated molecular weight of 78.7 kDa and a theoretical pI of 6.01. The amino acid analysis suggested that CitHex2 is rich in phenylalanine and tyrosine (15.8% of the total aa). Gene expression analysis showed that *CitHex*2 was expressed at all developmental stages but was differentially expressed during juvenile stages and more highly expressed in female tissues than in male tissues. 【Conclusion】 These findings show that *CitHex*2 is involved in the development of *C. italicus* and that it is also associated with reproduction.

Key words: *Calliptamus italicus*; hexamerin; cDNA; gene cloning; expression pattern

1 INTRODUCTION

Hexamerins are large hemolymph-proteins that have been discovered in all insect species studied so far. Typically, hexamerin subunits have masses of approximately 80 kDa, giving rise to a native molecule of approximately 500 kDa (Telfer and Kunkel, 1991). Hexamerins are transiently expressed during different developmental stages, where they are thought to act mainly as storage proteins that provide amino acids and energy during non-feeding periods (Telfer and Kunkel, 1991; Burmester, 1999a). Hexamerins have been reported to be synthesized in the fat body and exported into the hemolymph where they either accumulate or are taken up by the fat body and sequestered in storage granules in the cytoplasm (Hathaway *et al.*, 2009). When amino acids are needed, these stored proteins are broken down by the fat body so that the amino acids become accessible to meet the insect's metabolic demands.

Thus, hexamerins provide a buffer between resource availability and metabolic need for processes such as moulting, egg production, adult diapause, and caste formation (Chinzei *et al.*, 1990; Koopmanschap *et al.*, 1992; Pan and Telfer, 1996, 2001; Seo *et al.*, 1998; Hathaway *et al.*, 2009). Most insect hexamerins that have been identified to date to play a role in nutrient uptake and storage, but some are capable of binding the insect morphogenetic hormone juvenile hormone (JH) (Zhou *et al.*, 2006a).

Hexamerins resemble arthropod hemocyanins in terms of structure and amino acid sequence. Both hexamerins and hemocyanins belong to the same protein superfamily (Burmester, 2002). This protein superfamily includes hemocyanins, phenoloxidases, pseudo-hemocyanins, hexamerin receptors and hexamerins. Phenoloxidases are copper-containing enzymes involved in the melanin pathway (Decker and Terwilliger, 2000). Pseudo-hemocyanins (cryptocyanins) probably serve as storage proteins (Burmester, 1999b; Terwilliger *et al.*, 1999), and dipteran hexamerin receptors mediate the uptake of hexamerins by the larval fat body (Burmester and Scheller, 1996).

Hexamerins were initially thought to function solely as storage proteins. However, hexamerins may also transport hormones such as ecdysteroids (Enderle

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et al., 1983) and juvenile hormone (Braun and Wyatt, 1996), analogous to the function of vertebrate serum proteins. In termites, JH is classified as a primer pheromone (Henderson, 1998). The effects of JH on termite caste differentiation have been known for several decades (Henderson, 1998), and it has recently been demonstrated that JH induces the differentiation of the soldier caste from workers when its titres reach high enough levels (Mao *et al.*, 2005; Zhou *et al.*, 2006b). Studies have found storage hexamerins in many insects. These studies have been largely concentrated on the orders Diptera and Lepidoptera (Burmester, 1999a; Burmester, 2001). However, functions beyond a role in metamorphosis have also been reported in recent studies on hymenopteran hexamerins (Cristino *et al.*, 2010; Martins *et al.*, 2010, 2011). There have also been studies related to the function of storage hexamerins in Orthoptera, including *Locusta migratoria* (de Kor and Koomanschap, 1987; Ancsin and Wyatt, 1996; Braun and Wyatt, 1996) and *Schistocerca americana* (Hahn *et al.*, 2003). In *L. migratoria*, a high-molecular-weight protein (Mr 500 000), was isolated from the hemolymph, characterized, and identified as larval hemolymph protein (LHP) (Ancsin and Wyatt, 1996). Larval storage protein 1 (LSP1) and persistent storage protein (PSP) were identified in 1996 (Braun and Wyatt, 1996). In *S. americana*, a storage hexamerin, termed *S. americana* persistent storage protein (saPSP), was found and identified in 2003 (Hahn *et al.*, 2003). There is also evidence that at least some hexamerins are involved in the immune response. Surprisingly, hexamerins may also stimulate stem-cell proliferation (Blackburn *et al.*, 2004; Hakim *et al.*, 2007) and may play an intracellular role in transcription regulation (Martins *et al.*, 2011).

Within the Orthoptera, hexamerins display a remarkable diversity (Burmester, 1999a). Typically, Orthoptera possess distinct types of hexamerins that differ in terms of amino acid composition, evolutionary history and probably function. *Calliptamus italicus* has developed resistance to many chemical pesticides, and as it is an agricultural pest, it is important to study the physiology of this species. In this study, we isolated hexamerin subunit type 2 (Hex2) gene from *C. italicus* and cloned and sequenced the corresponding cDNAs. We then studied its expression at different developmental stages and in specific adult tissues. This study lays foundation for further study of the role of hexamerins throughout the grasshopper life cycle.

2 MATERIALS AND METHODS

2.1 Insects and sample preparation

C. italicus eggs were obtained from Shandong Agricultural University, Tai'an, Shandong. Eggs of *C. italicus* (cased in plastic and stored in vermiculite) were collected and held in a growth chamber at 27 ± 1℃ and 40% ± 5% relative humidity with a 14L: 10D photoperiod. Insects were fed with wheat seedlings *ad libitum* after hatching. To investigate the expression of hexamerin, a pool of 240 individuals were collected from this colony at different developmental stages, including embryos (5, 6, 7, 8, 9, and 10 d before hatching), nymphs (1st – 5th instars), and adult males and females. We collected the nymphs every two days during each nymphal instar. Nymphal instars were identified by the development of wings and individual nymphs were chosen randomly and removed from the colony every two days to assay total RNA. An equal number of males and females were dissected to obtain tissues and hemolymph samples. The adult tissues sampled included the prothorax, abdomen, hind femur, fat body, testis/ootheca, and midgut.

2.2 cDNA synthesis

Total RNA was extracted using RNAiso Plus (TaKaRa Biotechnology). RNA purity and concentration were determined by agarose gel electrophoresis and UV spectrophotometery, respectively. RNA was stored at −80℃. The first cDNA strand was synthesized using 1 μg of total RNA, 1 μL of oligo(dT)18 (0.5 μg/μL), 10 μL of 2 × TS Reaction Mix, and 1 μL of TransScript™ RT/RI Enzyme Mix (TransGenBiotech). The mixture (20 μL total volume) was incubated at 42℃ for 30 min and then at 85℃ for 5 min to inactivate the enzyme. Degenerate primers were designed based on the nucleotide sequences of the *LmiHex*2 (GenBank accession no.: FJ609739) and *RmiHex*2 (GenBank accession no.: AY923852). Gene specific primers were listed in Table 1. The PCR programme consisted of a 2 min denaturation step at 94℃, followed by 35 cycles of 94℃ for 30 s, 50℃ for 30 s, 72℃ for 1 min, and a 10 min chain extension at 72℃. Amplified fragments were visualized on 1% (w/v) agarose gel containing Goldview™ (ZOMANBIO) using a 2 kb DNA Ladder Trans 2K™ as a molecular weight marker (TransGen Biotech). Gene-specific primers were designed to complement the sequences of interest. 5′ RACE and 3′ RACE primers were listed in Table 1. The same primers were used with 3′ and 5′ RACE kits to complete the cDNA sequences (TaKaRa Biotechnology). PCR

Table 1 Primers used in this study

Primer name	Primer sequences (5′ – 3′)	Use
Hex2-F	TGGTBTCVTACTTYACCG	Amplification of *CitHex*2 fragment
Hex2-R	GCGCTTGATBTCRTTCTC	
GeneRacer 5′-R	CTGTTGAAGGACGAAGAGGA	Amplification of *CitHex*2 5′ end
GeneRacer 5n′-R	ATGTCTTCGGTGAAGTAGGA′	
GeneRacer 3′R	TCAACAACGCCCTGACCTTC	Amplification of *CitHex*2 3′end
GeneRacer 3n′-R	CCGTCGTCCGCGTCTTCATT	
Hex2-F	TCACTGTCGCTGTGCTGC	*CitHex*2 real-time PCR
Hex2-R	GTCGTATGCCTTCTGGATG	
β-actin-F	GCTCGTCCAAGGCATCAG	*β-actin* real-time PCR
β-actin-R	TCCATGTCGTCCCAGTTTG	

products were sequenced by a commercial service (Beijing Genomics Institute).

2.3 Sequence analysis

The complete cDNA sequence was translated into its putative amino acid sequence, which was then analysed using the NCBI BLAST database (http://www.ncbi.nlm.nih.gov/) to determine its phylogenetic relationship to other proteins in the same family. ExPASy (http://us.expasy.org/) was used to estimate the molecular weight and the amino acid composition of each protein and SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) (Bendtsen *et al.*, 2004) was used to determine the location of the putative signal sequence. Motif identification was carried out by ScanProsite (http://prosite.expasy.org/) (De Castro *et al.*, 2006). Homology modelling was performed by the SWISS-MODEL server (http://swissmodel.Expasy.org/) (Biasini *et al.*, 2014). The protein sequence alignment was created with ClustalX (Version 1.83) (Thompson *et al.*, 1997). The phylogenetic analysis was conducted using MEGA version 4 (Tamura *et al.*, 2007) by the neighbour-joining method with bootstrapping (500 replicates).

2.4 Quantitative real-time PCR (qRT-PCR)

Previously, we cloned the partial sequence of *β-actin* mRNA from *C. italicus* (data not shown). Therefore, *β-actin* mRNA was considered a house-keeping gene and was used as the internal control. Total RNA was extracted from the samples following the method described above. Three replicates were performed for each sample. qRT-PCR was performed using a SYBR® Premix (TaKaRa Biotechnology) and a MiniOpticon system (Bio-Rad). Primers used for qRT-PCR were listed in Table 1. The amplification protocol began with a 2 min pre-denaturation step at 95℃, followed by 40 cycles at 95℃ for 20 s and 60℃ for 30 s, and concluded with a melting curve protocol, which necessitated that the temperature be increased by 0.5℃ between each cycle from 60 to 95℃. We used the difference in threshold cycle ($-C_T$) values for statistical analysis of the qRT-PCR data. The mRNA levels of *Hex*2 were normalized relative to the control using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). Data are expressed as the means ± standard error (*SE*) from three independent experiments. Statistical analysis was performed using SPSS 11.5 software. A difference between the two sets of data was considered statistically significant at $P < 0.05$.

3 RESULTS

3.1 Sequence analyses of *Hex*2 from *C. italicus*

The cDNA of *Hex*2 cloned from *C. italicus* is 2 610 bp in length, containing a 2 022 bp open reading frame (ORF) and a 124 bp 3′ untranslated region (UTR). The sequence information was submitted to GenBank (Accession no.: JX204835), under the name *CitHex*2. The encoded protein is predicted to be 673 aa in length with an estimated molecular weight of 78.7 kDa and a theoretical pI of 6.01. Amino acid analysis suggests that the protein is composed of 19 different amino acids, with the following composition: 10.0% leucine, 8.0% glutamic acid, 7.9% phenylalanine, 7.9% tyrosine, 7.4% valine, and 7.0% alanine. *CitHex*2 is a moderately aromatic member of the hexamerin family with 7.9% phenylalanine and 7.9% tyrosine (15.8% of total aa), but with a low percentage of methionine (0.15%) (Fig. 1). A 19 aa putative signal peptide is predicted at the N-terminus. The encoded protein has three hemocyanin domains: hemocyanin-N (an all-alpha domain from aa 29 – 154), hemocyanin-M (a copper-containing domain between aa 158 – 424) and hemocyanin-C (an IgG-like domain between aa 430 – 664) (Fig. 2). The structures of hemocyanin-N, hemocyanin-M and hemocyanin-C were modelled using the on-line SWISS-MODEL service. Sequence comparisons showed that the CitHex2 has 84% and 80% nucleotide sequence identity with *L. migratoria manilensis* Hex2 and *Romalea microptera* Hex2,

respectively. The GenBank sequence of *CitHex*2 was identified by BLAST analysis against the entire *L. migratoria* genome sequence. The ORF of *CitHex*2 is distributed different contigs, and four contigs contain introns (Fig. 3). However, the length of the introns remains unknown due to the discontinuous genomic sequences.

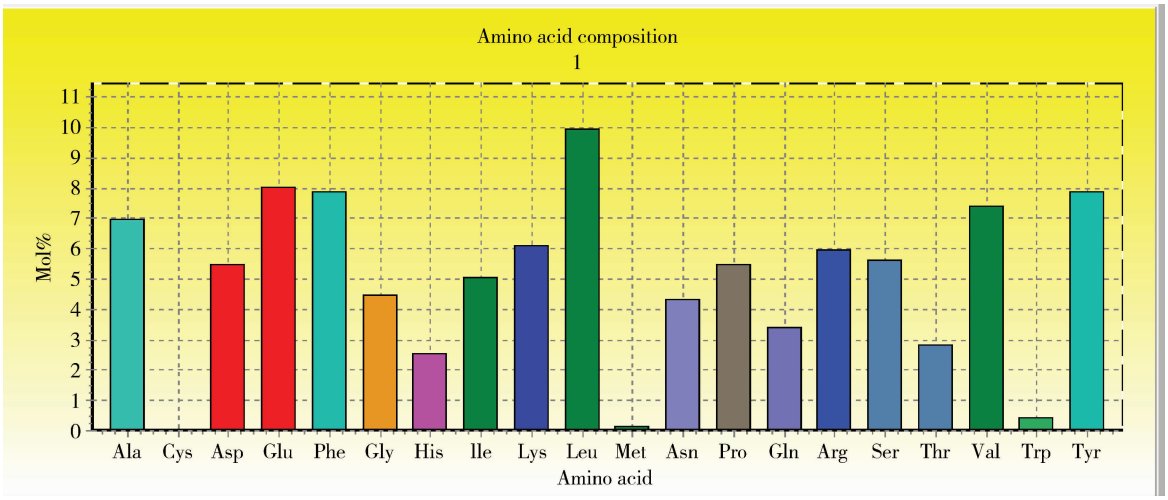

Fig. 1　Amino acid composition of the Hex2 protein from *Calliptamus italicus*

X-axis represents different amino acids and *Y*-coordinate represents the percentage of different amino acids.

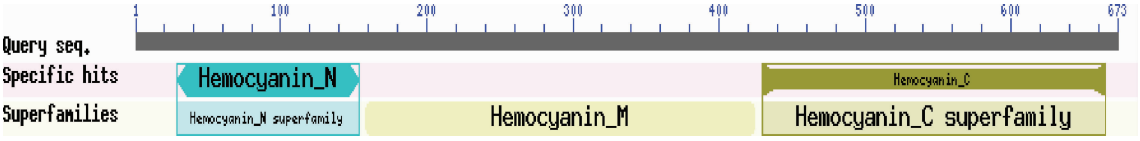

Fig. 2　Structural domain analysis of the amino acid sequence of Hex2 from *Calliptamus italicus*

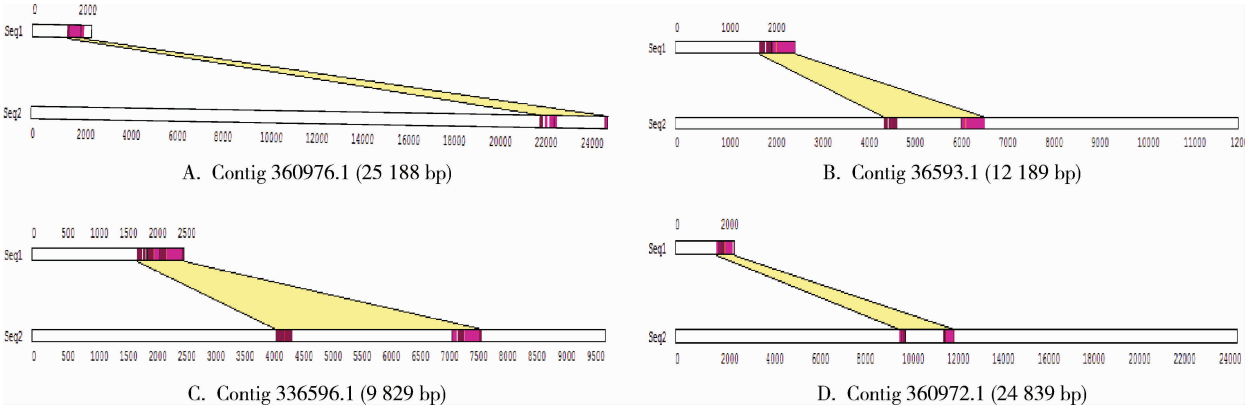

Fig. 3　Genomic structure of *Hex*2 from *Calliptamus italicus*

The red sections represent the gaps between the contigs, and the blank sections represent introns.

A multiple sequence alignment was constructed to compare CitHex2 to other five insect hemocyanin and hexamerin sequences obtained from the GenBank nucleotide database. Phylogenetic tree reconstructions were carried out using neighbour-joining based on the amino acid sequences. *Camponotus festinates* is found to be the outgroup. Orthoptera is a monophyletic group and divided into two lineages. The first clade consists of Orthoptera hexamerins with high support values (100). The second clade consists of Orthoptera hemocyanin with high support values (100). The Orthoptera hemocyanins form the sister group of the Orthoptera hexamerins (Fig. 4).

3.2　Expression analysis of *CitHex*2 in different tissues and at different developmental stages of *C. italicus*

The qRT-PCR data showed that *CitHex*2 mRNA was expressed in *C. italicus* at different levels throughout the lifecycle as well as in the adult tissues tested. During embryogenesis (5th – 10th day), the

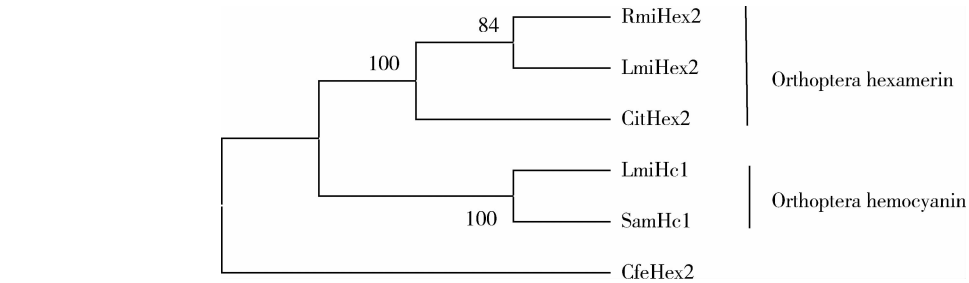

Fig. 4 Phylogenetic tree of the insect hemocyanin superfamily based on amino acid sequence (neighbour-joining method)

The numbers at the nodes represent bootstrap support values. The origin species of proteins and their accession numbers were as follows: RmiHex2: *Romalea microptera*, AAX14951.1; LmiHex2: *Locusta migratoria manilensis*, ACU78069.1; LmiHc1: *Locusta migratoria manilensis*, ADR82619.1; SamHc1: *Schistocerca americana*, AAC16760.1; CfeHex2 : *Camponotus festinatus*, CAB62053.1.

expression level of *CitHex*2 increased markedly, reaching a maximum value of 13.269 on the 8th day and then declining over subsequent days, and reaching a minimal value of 0.017. During the juvenile stages, the expression level of *CitHex*2 peaked on the 5th day of the 5th instar nymphal stage and then decreased, as observed during embryogenesis, to a minimal level at eclosion (Fig. 5). The expression level of *CitHex*2 in male and female tissues varied, with higher level in females than in males (Fig. 6).

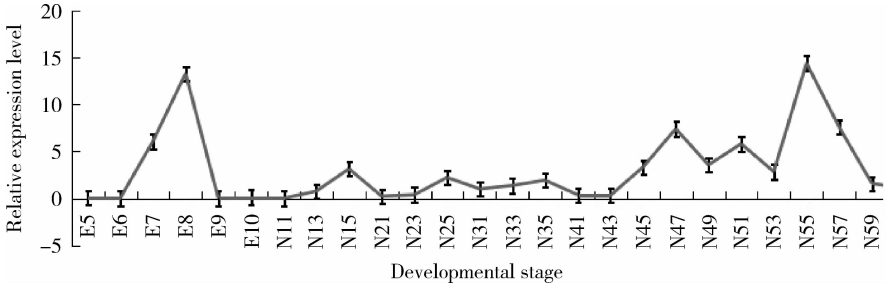

Fig. 5 Expression profiles of *CitHex*2 in different developmental stages of *Calliptamus italicus*

E5 – E10: 5th – 10th day of embryo; N11 – N15: 1st – 5th day of the 1st instar nymph; N21 – N25: 1st – 5th day of the 2nd instar nymph; N31 – N35: 1st – 5th day of the 3rd instar nymph; N41 – N49: 1st – 5th day of the 4th instar nymph; N51 – N511: 1st – 5th day of the 5th instar nymph. Three independent measurements were performed at each time point.

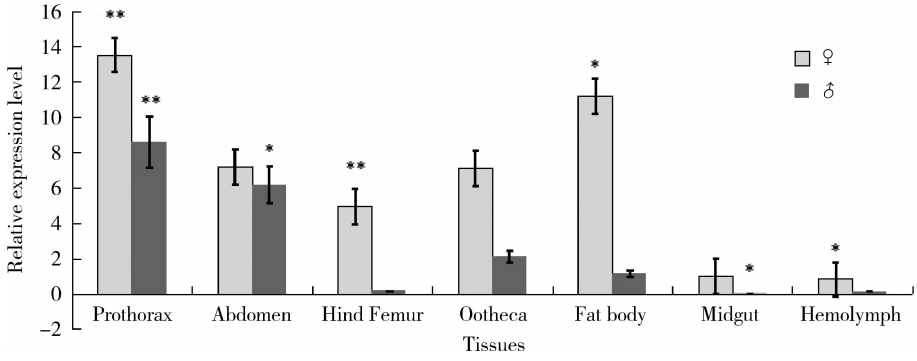

Fig. 6 Expression of *CitHex*2 in male and female adult tissues of *Calliptamus italicus*

Three independent experiments were performed for each tissue from each sex. * Significant difference at the 0.05 level by ANOVA analysis; ** Extremely significant difference at the 0.01 level by ANOVA analysis.

4 DISCUSSION

In this study, the Hex2 gene and its protein product were examined in *C. italicus* (Orthoptera) throughout its life cycle. A complete *Hex*2 coding sequence in *C. italicus* was isolated, cloned, and sequenced. Analysis of the amino acid composition suggests that this protein belongs to the arylphorin group because of its high aromatic amino acid content (15.8%).

Initially, hexamerins were considered to function solely as storage proteins to provide amino acids and energy during non-feeding periods such as during the moulting process (Munn *et al.*, 1967; Munn and Greville, 1969). This view is supported by the massive hexamerin accumulation that occurs during the

late nymphal or larval stages, when they may account for up to 50% of the soluble proteins. Hexamerins have also been discovered in the larvae of various holometabolous insects, where they are also considered to function primarily as storage proteins that provide energy and amino acids for metamorphosis (Munn *et al.*, 1967; Munn and Greville, 1969; Telfer and Kunkel, 1991; Burmester, 1999a; Burmester, 2014). However, little research has been carried out on Orthoptera other than *L. migratoria* (de Kor and Koomanschap, 1987; Ancsin and Wyatt, 1996; Braun and Wyatt, 1996) and *S. americana* (Ancsin and Wyatt, 1996). In this study, we identified and compared the expression of *Hex*2 from *C. italicus* by qRT-PCR. The results showed that *Hex*2 mRNA was expressed at different levels during the life cycle and in different tissues. There was a steady increase in expression level during the juvenile stages. The expression level peaked at the 5th instar nymphal stage and then rapidly declined at eclosion. This pattern supports the categorization of *C. italicus* Hex2 as an arylphorin, as these proteins also show a significant accumulation of their mRNAs before eclosion. This pattern may be caused by the regulation of hexamerin expression by ecdysone and juvenile hormone (Jones *et al.*, 1988, 1993; Manohar *et al.*, 2010; Burmester, 2014).

Total protein levels were found to be higher in females than in males. Within the Lepidoptera, MRSP and MMRSP are more abundant in females than in males (Webb and Riddiford, 1988), and it had been suggested that they support female reproduction and egg development by enhancing the pool of sulfur-containing amino acids used for vitellogenesis (Pan and Telfer, 1996). Therefore, the higher expression of *Hex*2 in female tissues indicates that *CitHex*2 might be involved in *C. italicus* reproduction. In the present study, we found that the Hex2 gene was expressed at a high level in the prothorax. Within the Lepidoptera, RNA-seq data indicate that Hex5 was expressed in the head and possibly the brain of larvae, pupae and adults. This result suggests a specific but currently unknown function of hexamerin proteins in the insect nervous system (Burmester, 2014). Further research is required to confirm these expression patterns. Munn (1971) proposed that the high proportion of aromatic amino acids of Hex2 is related to the demand for these components during sclerotization of the cuticle (Cristino *et al.*, 2010; Martins *et al.*, 2010, 2011). Therefore, further analyses on *Hex*2 expression in female *C. italicus* would help to develop a better understanding of the function of this protein during development.

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意大利蝗存储蛋白 Hex2 基因的克隆与表达分析

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摘要：【目的】昆虫存储蛋白（hexamerin）是在昆虫体内独立发生普遍存在的一种特异性淋巴蛋白，在昆虫的生长发育过程中起重要作用。【方法】克隆意大利蝗 *Calliptamus italicus* 的存储蛋白 Hex2 基因，并利用 qRT-PCR 方法分析其在不同组织和不同发育阶段的表达模式。【结果】克隆到意大利蝗存储蛋白 Hex2 基因 *CitHex*2，其 cDNA 全序列长 2 610 bp，开放阅读框（ORF）长为 2 022 bp，3′非翻译区（UTR）长为 124 bp，碱基序列与其他蝗虫的 Hex2 基因核苷酸一致性为 80% ~84%。*CitHex*2 编码 673 个氨基酸，预测分子量和等电点分别为 78.7 kDa 和 6.01。氨基酸分析表明，CitHex2 富含较高的芳香族氨基酸，其中苯丙氨酸和酪氨酸共占 15.8%。定量分析结果表明，*CitHex*2 在意大利蝗的整个发育阶段都有表达，且在每个龄期的蜕皮前后均有表达量的变化；在检测的所有组织中，雌性个体的表达量较高。【结论】*CitHex*2 参与意大利蝗的发育过程，与其生殖有关。

关键词：意大利蝗；存储蛋白；cDNA；基因克隆；表达模式

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